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Arginine transport in *Streptococcus lactis* is catalyzed by a cationic exchanger

(secondary solute transport/arginine:ornithine antiport)

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ABSTRACT **_Streptococcus lactis_ metabolizes arginine via the arginine deiminase pathway to ornithine, CO_2, NH_3, and ATP. The translocation of arginine and ornithine has been studied using membrane vesicles of galactose/arginine-grown cells of _S. lactis_ fused with cytochrome _c_ oxidase proteoliposomes by the freeze/thaw–sonication procedure earlier described. In the presence of reduced cytochrome _c_ the fused membranes rapidly accumulate ornithine. Addition of arginine releases accumulated ornithine. Rapid uncoupler-insensitive exchange between external arginine and internal ornithine is seen at rates that are at least 60-fold higher than the rate of protonmotive force-driven arginine translocation. This arginine:ornithine exchange activity was reconstituted in proteoliposomes after solubilization of _S. lactis_ membranes with octyl β-D-glucopyranoside. These proteoliposomes catalyze a one-to-one exchange between arginine and ornithine. The arginine:ornithine exchange system is the first exchange system for cationic metabolites found in bacteria. Translocation of arginine via this system does not require metabolic energy obtained by arginine metabolism.**

Group N streptococci (e.g., *Streptococcus lactis*, *Streptococcus cremoris*, and *Streptococcus lactis* subsp. *diacetylactis*) are limited in their ability to ferment compounds other than sugars. An exception to this is arginine metabolism by *S. lactis* and *S. lactis* subsp. diacetylactis (1). This metabolism of arginine to ornithine, ammonia, and carbon dioxide via the arginine deiminase pathway (for review, see ref. 2) is coupled to ATP synthesis, and ornithine is excreted (1).

The arginine deiminase pathway includes three enzymes: (*i*) arginine deiminase (L-arginine transferase, EC 3.5.3.6) catalyzes the conversion of arginine into citrulline and ammonia, (*ii*) ornithine carbamoyltransferase (EC 2.1.3.3) catalyzes the phosphorolysis of citrulline, yielding ornithine and carbamoylphosphate, and (*iii*) carbamate kinase (ATP: carbamate phosphotransferase, EC 2.7.2.2) catalyzing the transfer of phosphate from carbamoylphosphate to ADP, yielding ATP, ammonia, and carbon dioxide. The properties of these enzymes have been described for *S. lactis* (1, 2). The physiology of arginine metabolism has been well characterized, but less information is available about the mechanism of arginine transport. High rates of arginine metabolism have been reported (1), suggesting that arginine is translocated into cells via a transport system with a large capacity. In this paper we describe a unique transport system for arginine in *S. lactis*. This transport system catalyzes a one-to-one exchange between arginine and ornithine. The arginine: ornithine antiporter can be extracted and reconstituted into proteoliposomes in a functional state.

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MATERIALS AND METHODS

Growth of Strains and Isolation of Membrane Vesicles. *S. lactis* strain ML3 was grown anaerobically on MRS broth (3) in the presence of 110 mM galactose and 20 mM L-arginine at 30°C. Membrane vesicles of *S. lactis* ML3 were prepared by osmotic lysis as described (4) and stored in liquid nitrogen until use.

Reconstitution of Cytochrome _c_ Oxidase in Liposomes. Cytochrome *c* oxidase, isolated from beef heart mitochondria (5) was reconstituted into liposomes containing acetone/ether-washed *Escherichia coli* phospholipids (6) by dialysis as described (7, 8).

Fusion of cytochrome *c* oxidase proteoliposomes with *S. lactis* membrane vesicles. *S. lactis* membrane vesicles (100 μl, 1 mg of protein) and cytochrome *c* oxidase proteoliposomes (500 μl, 10 mg of phospholipid, 1.15 nmol of cytochrome *c* oxidase) were mixed and fused by freeze/thaw–sonication as described (7–9).

Transport Assay. Membrane vesicles were diluted by a factor of 30–40 at pH 6.0 with 50 mM potassium phosphate (KP_i) and 500 μM ornithine, unless stated otherwise. After 2-hr incubation at 21–23°C, membrane vesicles were pelleted by centrifugation (48,000 × *g* for 30 min at 4°C). After resuspension in the same buffer (20–25 mg of protein/ml), small aliquots (4 μl) were rapidly diluted into 50 mM KP_i, pH 6.0 (400 μl), supplemented with labeled substrate. At indicated time intervals samples were filtered (0.45-μm pore size, Millipore, cellulose nitrate) and washed twice with 0.1 M LiCl as described (7, 8). Radioactivity retained on the filters was measured as described previously (7, 8). Uptake of labeled amino acids by vesicles containing cytochrome *c* oxidase was done as described (7–9).

Reconstitution. Membranes (0.6 mg protein) were solubilized with 1.25% (wt/vol) octyl β-D-glucopyranoside (10) in the presence of 18 mg of a mixture of egg phosphatidylcholine and acetone/ether-washed *E. coli* phospholipid (6) 1:3 (wt/wt)/20% glycerol (vol/vol) (11)/2 mM ornithine/50 mM KP_i, pH 6.4, in a final volume of 5 ml. The suspension was incubated for 20 min on ice and centrifuged for 1 hr at 48,000 × *g* at 4°C. To the clarified supernatant (4.5 ml), 300 μl of the phospholipid mixture (50 mg/ml) was added, and the suspension was kept on ice for 10 min. The solution was dialyzed for 4 hr against a 1000-fold volume of 50 mM KP_i, pH 6.4, at 4°C. Dialysis was continued overnight at 4°C against a 1000-fold volume of the same buffer. Proteoliposomes were collected by centrifugation (280,000 × g_{max}, 45 min, 4°C) and washed once with 50 mM KP_i, pH 6.4.

Other Analytical Procedures. Protein was determined as described (12). An internal volume of 8 μl/mg of protein was used for fused membranes (7).

Abbreviations: Δp, transmembrane electrochemical potential; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; Ph_4P^+, tetraphenylphosphonium ion; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

Materials. Octyl β-D-glucopyranoside, horse heart cytochrome *c*, egg phosphatidylcholine and crude *E. coli* phospholipids were obtained from Sigma. L-[2,3-^{3}H]Ornithine (1.1 TBq/mmol) was obtained from New England Nuclear. L-[U-^{14}C]Arginine (11 TBq/mol) was purchased from Amersham. All other materials were obtained from commercial sources with the highest grade of purity available.

RESULTS

Exchange Between Arginine and Ornithine. Membrane vesicles of *S. lactis* ML3, prepared from galactose/arginine-grown cells were fused with proteoliposomes containing beef heart mitochondrial cytochrome *c* oxidase by means of the freeze/thaw–sonication technique (7–9). Fused membranes were allowed to accumulate [^{3}H]ornithine in the presence of ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD)/cytochrome *c* (Fig. 1). After 20 min [^{14}C]arginine was added, which resulted in a rapid accumulation of arginine with concomitant release of ornithine (Fig. 1). The rate of arginine uptake was stimulated ≈60-fold when ornithine was present at the inside.

In another experiment, membrane vesicles were preloaded with 200 μM ornithine and diluted by a factor of 50 into a buffer containing [^{14}C]arginine or [^{3}H]ornithine (Fig. 2). A transient accumulation of arginine and ornithine was observed under those conditions. The time course of uptake was not affected by the presence of either 10 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (CCCP) or the combination of 1 μM valinomycin and 0.1 μM nigericin (data not shown). Low levels of uptake were found when membrane vesicles were used that were not loaded with ornithine (Fig. 2).

Kinetic Properties of Exchange. When initial rates of arginine uptake were examined within an amino acid concentration of 0.3–40 μM and 500 μM ornithine was inside the vesicles, a Michaelis constant for arginine entry transport (K_t^{entry}) of 1.1 μM and a maximal velocity (V_{max}) of 28.5 nmol arginine/min·mg of protein was found (Table 1). The initial external ornithine concentration was <2 μM in this experiment. Under similar conditions homologous exchange of ornithine showed a K_t^{entry} of 8.4 μM and a V_{max} of 29.4 nmol/min·mg of protein (Table 1). Exchange of L-ornithine was competitively inhibited by L-arginine with a K_i of 2.1 μM. On the other hand, arginine uptake was competitively inhibited by ornithine with a K_i of about 9 μM. Under conditions that the dilution factor was kept constant, an increase in the maximal rate of arginine uptake was observed with increasing amount of ornithine at the inside (Fig. 3). K_t^{entry} for arginine remained largely unaffected. Strong inhibition of arginine uptake was observed when the external ornithine concentration after dilution exceeded 10 μM (data not shown), most likely due to competitive inhibition of arginine uptake. The apparent K_t^{exit} value for ornithine was estimated indirectly from the rate of arginine uptake under conditions in which the internal ornithine concentration was varied (Fig. 3), yielding a value of about 73 μM (*Inset* Fig. 3).

Substrate Specificity. The substrate specificity for the exchanger at the outside was determined under conditions that 500 μM ornithine was present at the inside (outside ≈4 μM). Rapid exchange was observed when [^{14}C]arginine, [^{3}H]ornithine (Fig. 2), or [^{14}C]lysine (data not shown) was added to the outside. [^{14}C]Citrulline, [^{14}C]alanine, and [^{14}C]leucine were not accumulated. The substrate specificity for the exchanger at the inside was determined by preloading membrane vesicles with 200 μM of different ornithine analogues. Preloaded membrane vesicles were diluted by a factor of 100 into a medium containing 0.4 μM [^{14}C]arginine. The rate of arginine uptake decreased in the following order: L-2,5-diaminopentanoic acid (L-ornithine) > L-2,6-diaminohexanoic acid (L-lysine) > D-2,5-diaminopentanoic acid (D-ornithine) >> L-2,4-diamino-*n*-butyric acid, D,L-2,3-diami-

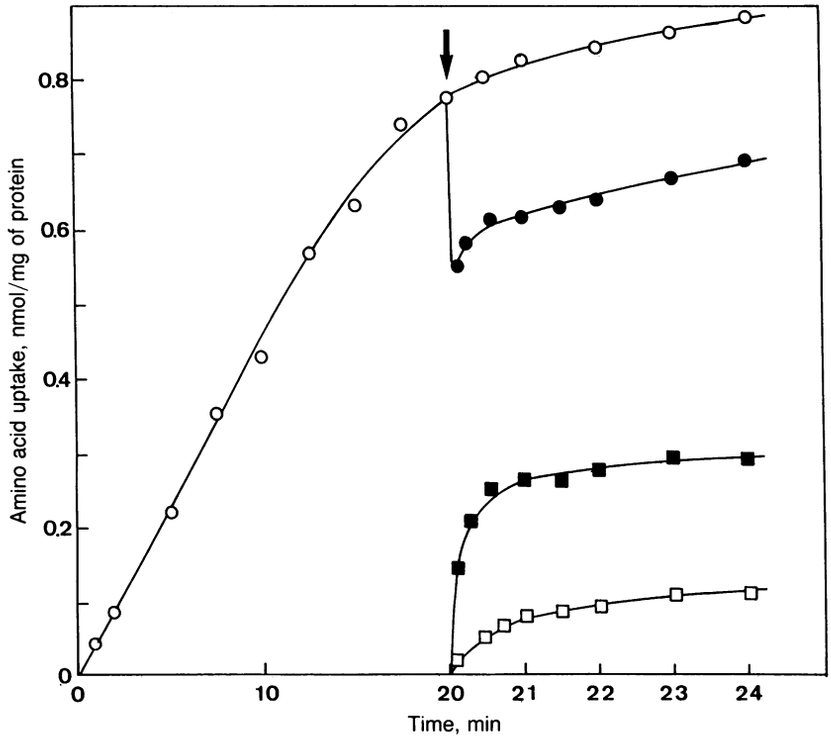

FIG. 1. Effect of arginine on ornithine uptake by membrane vesicles of *S. lactis* fused with cytochrome *c* oxidase proteoliposomes. Fused membranes (0.35 mg of protein/ml) were incubated in a solution containing 50 mM KP_i (pH 6.0), 200 μM TMPD, 20 μM cytochrome *c*, and 10 nM nigericin. Thirty seconds after the addition of 15 mM ascorbate, 4.2 μM [^{3}H]ornithine (○) was added, and uptake was assayed as described. In a parallel experiment 2.1 μM [^{14}C]arginine was added as indicated by the arrow, and uptake and release of, respectively, arginine (■) and ornithine (●) were followed. Uptake of 2.1 μM [^{14}C]arginine (□) is also shown in the absence of ornithine.

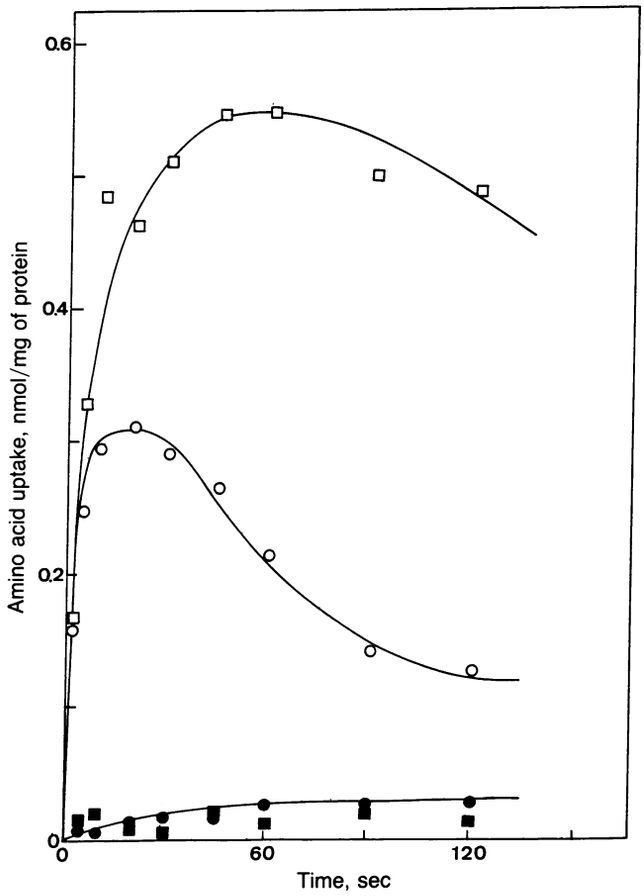

FIG. 2. Ornithine and arginine uptake by ornithine-loaded membrane vesicles. Membrane vesicles incubated in the presence (open symbols) or absence (closed symbols) of 200 μM ornithine were resuspended in 50 mM KP_i, pH 6.0, containing either [^{3}H]ornithine (4 μM final concentration) (□, ■) or [^{14}C]arginine (0.75 μM) (○, ●). A final protein concentration of 0.4–0.5 mg/ml was used.

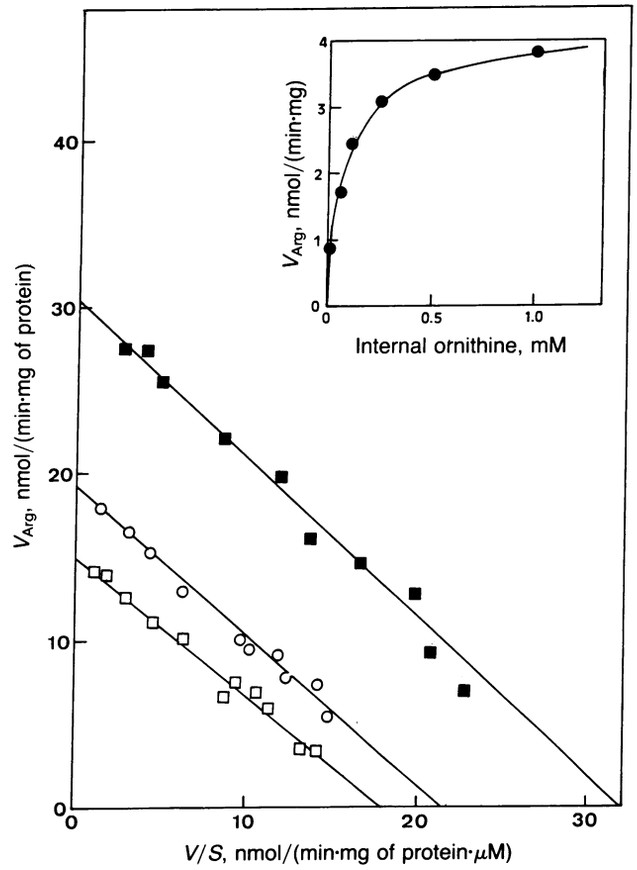

FIG. 3. Kinetics of arginine:ornithine exchange. Membrane vesicles were loaded with 50 μM (□), 100 μM (○), or 500 μM (■) ornithine, and diluted in 50 mM KP_i, pH 6.0, supplemented with 0.3–40 μM [^{14}C]arginine. Initial rates were estimated from the uptake of arginine after 4-sec incubation. (*Inset*) Relation between the initial rate of arginine uptake and the internal ornithine concentration. Membrane vesicles were loaded with ornithine at concentrations of 0–1 mM as described. Membrane vesicles were diluted by a factor of 50 in 50 mM KP_i, pH 6.0, containing 0.37 μM [^{3}H]arginine.

nopropionic acid (data not shown). Efflux of L-norvaline, an analogue lacking the side-chain amine group did not drive arginine uptake.

Reconstitution of Exchange in Proteoliposomes. Membranes were solubilized and subsequently reconstituted into proteoliposomes by detergent dialysis. An extremely rapid accumulation of arginine was observed when proteoliposomes preloaded with 200 μM ornithine were diluted into a solution containing [^{14}C]arginine (Fig. 4). Uptake of arginine was not affected by the combination of the ionophores nigericin (0.1 μM) and valinomycin (1 μM) (Fig. 4). Hardly any uptake of arginine was observed when proteoliposomes were used that were not loaded with ornithine (Fig. 4). Accumulated arginine was rapidly chased by a 50-fold excess of unlabeled arginine (data not shown).

Table 1. Kinetic parameters of arginine:ornithine and ornithine:ornithine exchange

Translocated amino acid	Internal ornithine, μM	K_t, μM	V_{max}, nmol·(min·mg of protein)$^{-1}$	K_i^{app}, μM
Arginine	200	0.8	20.1	9.1 (Orn)
	500	1.1	28.5	ND
Ornithine	200	3.7	19.3	ND
	500	8.4	29.4	2.1 (Arg)

Kinetic constants and inhibitor constants (K_i^{app}) were estimated from the amount of labeled amino acid accumulated in 4 sec. Uptake was done as described in the legend for Figs. 2 and 3. Ornithine-loaded membrane vesicles were diluted by a factor of 50 into 50 mM KP_i, pH 6.0, supplemented with [^{14}C]arginine (0.3–40 μM) or [^{3}H]ornithine (2–50 μM). K_i^{app} of competitive inhibition was determined by varying the substrate concentration in the presence of a fixed inhibitor concentration. Arginine and ornithine were used at a concentration of 3 and 10 μM, respectively. ND, not determined.

Stoichiometry of Exchange. The stoichiometry of exchange was determined directly with the reconstituted exchanger at 15°C to allow an accurate estimation of the stoichiometry in the first 20 sec. Proteoliposomes were loaded with 20 μM [^{3}H]ornithine and diluted by a factor of 50 into a buffer containing 0.375 μM [^{14}C]arginine (Fig. 5). In a parallel experiment, ornithine-loaded membranes were diluted into arginine-free buffer. A correction was made for ornithine-independent arginine uptake by diluting nonloaded proteoliposomes into a buffer containing labeled arginine. The exchange stoichiometry was calculated from the net amount of arginine-stimulated release of ornithine, and the net amount of ornithine-stimulated uptake of arginine. The data presented in Fig. 5 (see also *Inset*) indicates that the transporter catalyzes a one-to-one exchange between arginine and ornithine.

DISCUSSION

This paper reports an antiporter of cationic metabolites in bacteria. This antiport transport system catalyzes a one-to-

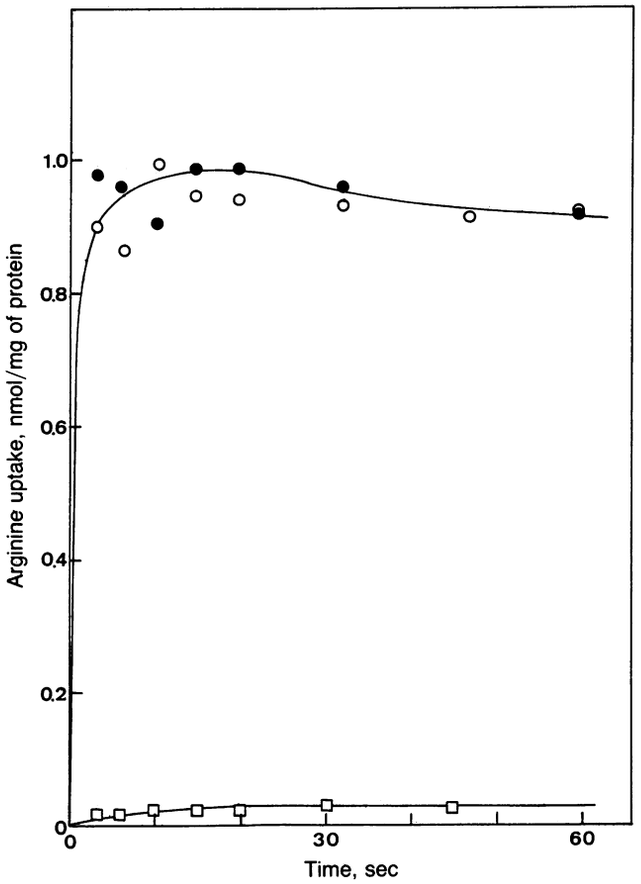

FIG. 4. Reconstitution of arginine:ornithine exchange in proteoliposomes. Proteoliposomes were loaded with 200 μM ornithine and diluted by a factor of 50 in 50 mM KP_i, pH 6.0, containing 0.75 μM [^{14}C]arginine in the absence (●) or presence of 0.1 μM nigericin and 1 μM valinomycin (○); arginine uptake by proteoliposomes incubated in the absence of ornithine (□) is also shown. Uptakes were done at 25°C. Solubilization and reconstitution were done as described. Proteoliposomes were used at a final concentration of 0.06–0.1 mg of protein/ml.

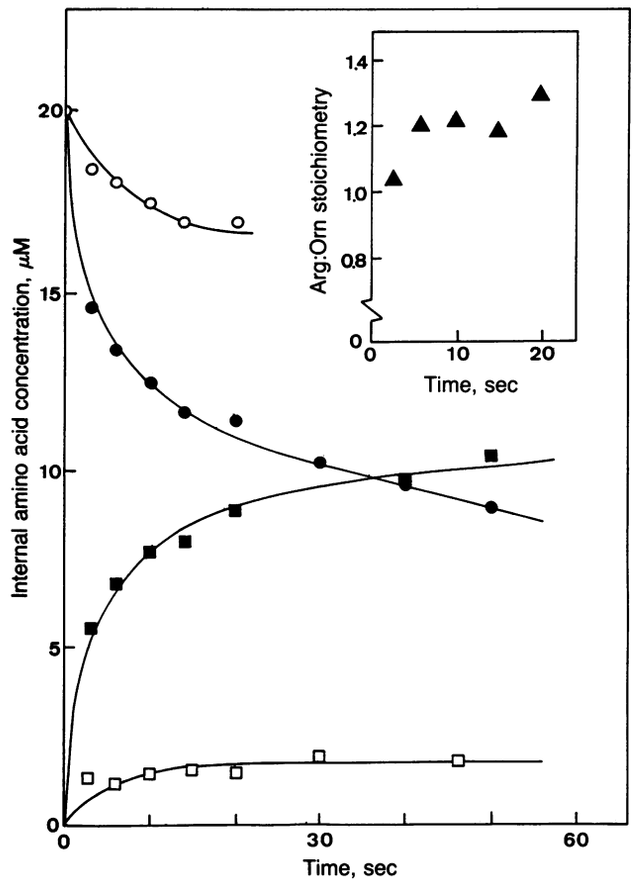

FIG. 5. Stoichiometry of arginine:ornithine exchange in proteoliposomes. Proteoliposomes were loaded with 20 μM [^{3}H]ornithine and diluted by a factor of 50 in 50 mM KP_i, pH 6.0. [^{3}H]Ornithine efflux in the presence (●) or absence (○) or 0.375 μM [^{14}C]arginine. [^{14}C]Arginine uptake by proteoliposomes loaded (■) or nonloaded (□) with ornithine. Uptakes were done at 15°C. (*Inset*) Arginine:ornithine stoichiometry calculated from ornithine-stimulated arginine uptake and arginine-stimulated ornithine efflux. Experimental procedures were as described in the legend for Fig. 4 and in *Materials and Methods*.

one heterologous exchange between arginine and ornithine, or homologous exchange of ornithine or arginine (unpublished work). Because arginine and ornithine are both positively charged in the neutral pH range, exchange will be electroneutral. Consequently, the rate of exchange was not altered by the addition of uncouplers or the imposition of a Δp (unpublished data). Electroneutral exchange has been demonstrated for the phosphate:sugar-phosphate antiporter of *S. lactis* ATCC 7962 (13). Arginine:histidine antiport in vacuolar membrane vesicles of the yeast *Saccharomyces cerevisiae* also appears to be electroneutral (14). In contrast, the mitochondrial ATP/ADP (15) and glutamic acid/aspartate (16) exchangers are electrogenic. The initial rates of arginine:ornithine exchange indicated are most likely an underestimation of real rates. Rates were determined from the amount of arginine accumulated within 4 sec. Under those conditions internal arginine concentrations already exceed the K_t for arginine uptake, thereby slowing down ornithine release (Fig. 5)—most likely as a result of competition for a single binding site. In intact cells, arginine is converted into ornithine (1), which enables a continuation of arginine uptake in exchange for ornithine. Although it is difficult to make an exact estimate, rates of up to 1 μmol of arginine/(min·mg of protein) can be found in intact cells loaded with ornithine (B.P. and A.J.M.D., unpublished data). Estimation of the maximal velocity in proteoliposomes, calculated from the amount of arginine accumulated within 3 sec, indicates a V_{max} that will be far above 55 nmol/(min·mg). Experiments have to be performed at low temperatures in order to determine real V_{max} values. Δp-driven arginine uptake occurs with a much lower rate than arginine:ornithine exchange, although the K_t for Δp-driven arginine transport (unpublished work) is similar to the K_t found for the exchange reaction. However, by these kinetic data alone the existence of a second transport system for arginine cannot be excluded. The arginine:ornithine antiporter has to be isolated and purified in order to test whether this transport system is also capable of facilitating Δp-driven translocation of arginine. In order to initiate arginine exchange for internal ornithine, cells require an alternative transport system for ornithine. This requirement is fulfilled by the lysine transport system, which also catalyzes H^+—ornithine symport with a low affinity, e.g., 56 μM (unpublished data). In this respect, cells are able to maintain a high internal ornithine pool under conditions of arginine metabolism. Because ornithine is not further metabolized (17), exchange for arginine can occur at high rates.

Crow and Thomas (1) demonstrated that arginine deiminase and ornithine transcarbamylase are induced when in continuous cultures glucose limitation occurs. Under those conditions arginine is converted into ornithine. In addition to these two cytoplasmic enzymes, induction of the arginine:

ornithine antiporter can be demonstrated in a similar experiment (B.P. and A.J.M.D., unpublished work). Moreover, the antiporter is absent in *S. cremoris*, a variety of *S. lactis* that is unable to ferment arginine. The arginine:ornithine antiporter can also be found in a number of group D (*S. faecalis* DS5) and viridans streptococci (*S. sanguis* 12 and *S. milleri* RG1-type 2) (B.P. and A.J.M.D., unpublished work) that are able to utilize arginine, emphasizing its important role in arginine metabolism.

The results clearly demonstrate that in *S. lactis* an arginine:ornithine antiporter is operational. The driving force for arginine uptake is supplied by the concentration gradient of ornithine, formed during arginine metabolism. In this way energy obtained from the metabolism of arginine can be saved for biosynthesis. This strategy might also be found in other bacteria that excrete ornithine during arginine metabolism (2).

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